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**Are all bony fishes oxygen regulators? Evidence for oxygen regulation in a putative oxygen conformer, the swamp eel *Synbranchus marmoratus***

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### ABSTRACT

This study investigated the oxygen consumption of the putative oxygen conformer marbled swamp eel *Synbranchus marmoratus* during progressive hypoxia. Earlier studies have not reached agreement on whether *S. marmoratus* is a conformer or regulator. Our results support the view that *S. marmoratus* is an oxygen regulator, like most bony fishes.

Most bony fishes (Teleostii) are hypothesized and shown to be oxygen regulators (Ultsch *et al.*, 1981; Steffensen, 2006), whereby they maintain their oxygen consumption during progressive hypoxia until the ambient partial pressure of oxygen becomes too low and the fish reach their critical oxygen tension,  $pO_{2crit}$  (Tang, 1933). Below  $pO_{2crit}$ , aerobic metabolic rate cannot be maintained and the fish becomes an anaerobic oxygen conformer as its oxygen consumption decreases with environmental oxygen. A few species of teleosts have been suggested to be oxygen conformers. These species, which include the marbled swamp eel *Synbranchus marmoratus* Bloch 1795, in addition to inanga *Galaxias maculatus* (Jenyns 1842), sturgeons

*Acipenser* spp., walking catfish *Clarias batrachus* (Hamilton 1822), and plainfin midshipman *Porichthys notatus* Girard 1854 (Singh & Hughes, 1971; Graham & Baird, 1984; Urbina *et al.*, 2012; Tripathi *et al.*, 2013; Craig *et al.*, 2014; LeMoine *et al.*, 2014), appear to exhibit declining oxygen consumption rates during progressive hypoxia. In this study we challenge the assumption that aerobic oxygen conforming fishes exist (Ultsch *et al.*, 1981b; Steffensen, 2006), by testing the ability of the *S. marmoratus*, one of the few reported oxygen conformers, to maintain oxygen consumption during exposure to progressive hypoxia.

Fish regulate different physiological parameters to maintain their oxygen consumption to meet the oxygen demand determined by their metabolism (Jones *et al.*, 1970; Jones, 1971). When exposed to moderate hypoxia most exclusive water-breathing fish increase transport of water over the gills (Randall & Shelton, 1963; Wood & Shelton, 1980; McKenzie *et al.*, 1991) by increasing ventilation frequency and volume (Steffensen *et al.*, 1982). When exposed to severe hypoxia, a decrease in heart rate (bradycardia) will occur with a concurrent increase in blood pressure that increases recruitment of gill lamellae as blood flows through the gills (Randall & Shelton, 1963; McKenzie *et al.*, 2007, 2009). Changes in oxygen binding properties of fish blood help facilitate oxygen uptake and delivery to tissues during hypoxia by increasing the oxygen affinity and carrying capacity of haemoglobin (Bohr *et al.*, 1904; Root, 1931; Berenbrink, 2007; Rummer *et al.*, 2013). In combination, these factors are instrumental in maintaining the capacity of an oxygen regulating fish to consume oxygen during hypoxia. By comparison, an oxygen conforming fish should lack one or more of these capabilities. However, even fish without haemoglobin (as well as salamanders, which are not capable of aquatic ventilation) have been shown to be aquatic oxygen regulators (Holeton, 1970; Hemmingsen *et al.*, 1972; Ultsch & Duke, 1990). While a few species, such as *Acipenser naccarii* Bonaparte

1836 (McKenzie *et al.*, 2001, 2007) and hellbender salamanders (Ultsch & Duke, 1990), may be oxygen conforming under certain conditions (*e.g.*, when prevented from swimming, thereby limiting convective oxygen transport), this situational dependency could mask the true ability of a species to regulate oxygen consumption and lead to erroneous conclusions of oxygen conforming. The purpose of this study was to affirm or dismiss oxygen conforming as a respiratory strategy in *S. marmoratus*; it has been reported to be both an oxygen conformer (Graham & Baird, 1984; Graham, 1997) and an oxygen regulator (Bicudo & Johansen, 1979).

A total of 12 marbled swamp eels (body mass, mean  $\pm$  S.D. =  $224 \pm 4.7$  g, range 160–328 g) were collected from natural ponds near Rio Claro, Brazil. All fish were caught by hand net and immediately transferred to the Rio Claro UNESP holding facility where individual fish were placed in a separate plastic flow-through aquarium. Being facultative air breathers, they had access to air when held in the aquaria and the flow-through water temperature was kept at a 23–26°C diurnal temperature variation similar to the collection ponds. All fish were kept under a natural 12 h light:12 h dark photoperiod.

Oxygen uptake ( $\dot{M}_{O_2}$ ) during experimentation was measured using intermittent-flow respirometry (Steffensen, 1989; Svendsen *et al.*, 2016a, 2016b) controlled using AquaResp V1 (University of Copenhagen; [www.aquaresp.com](http://www.aquaresp.com)). Oxygen was measured using PreSens (Fibox 3, Presens; [www.presens.de](http://www.presens.de)) oxygen sensors. Two identical 1.5 l respirometers were constructed, using capped PVC tubing with an access lid, a recirculation loop for mixing and a flush chimney. The tubing was similar to tubing that fish voluntarily occupied in the holding facilities. Eheim ([www.eheim.com](http://www.eheim.com)) aquarium pumps (model 1046,  $5 \text{ l min}^{-1}$ ) were used for recirculation and flushing, and the two respirometers were submerged in a 100 l tank with temperature-controlled and aerated fresh water. Water temperature was maintained at  $26.0 \pm$

0.1°C (mean  $\pm$  hysteresis), and  $\dot{M}_{O_2}$  was measured using 600 s flush, 60 s wait, and 600 s measurement (closed respirometer) loops. The fish had no access to air in the respirometers and they were allowed to acclimate in the respirometers for at least 12 h during which time oxygen uptake rate was recorded and used to determine the standard metabolic rate (SMR) (Beamish, 1964b). Following SMR recordings, hypoxia was induced to determine  $pO_{2crit}$ . This was done by pausing the intermittent flushing of the respirometer and allowing the fish to reduce the available oxygen in the respirometer from *c.* 18 to 1.3 kPa. Inducing progressive hypoxia using this method increases CO<sub>2</sub> levels as the fish respire (Malte *et al.*, 2016; Snyder *et al.*, 2016). A degree of hypercapnia must therefore have been present in the water during our experiments (Keys, 1930b; Piiper *et al.*, 1971; Hughes, 1973; Piiper & Scheid, 1984), but due to the fast induction of hypoxia this is not believed to have affected our results (Regan & Richards, 2017). Additionally, the previous studies of hypoxia tolerance in *Synbranchus marmoratus* induced hypoxia using similar methods (Bicudo & Johansen, 1979; Graham & Baird, 1984), making our results directly comparable with these previous studies (Figure 1). The SMR was calculated by fitting a double Gaussian distribution to all measured  $\dot{M}_{O_2}$  data as described in Steffensen *et al.* (1994; Chabot *et al.*, 2016). The critical oxygen tension was established using a protocol similar to that of Yeager and Ultsch (1989) but employing assumptions as in Chabot and Claireaux (2016), thus finding the intersection between constant SMR and a regression line representing oxygen conformation.

After introduction to the respirometers, fish showed elevated  $\dot{M}_{O_2}$  up to 5 h. The SMR of *S. marmoratus* in normoxia was  $1.18 \pm 0.14$  mmol O<sub>2</sub> h<sup>-1</sup> kg<sup>-1</sup> (mean  $\pm$  S.D). During progressive hypoxia exposure the fish were able to maintain SMR or higher  $\dot{M}_{O_2}$  until a critical partial pressure ( $pO_{2crit}$ ) of  $4.65 \pm 1.59$  kPa (mean  $\pm$  S.D) (Figure 1). The elevated  $\dot{M}_{O_2}$  observed at moderate hypoxia is likely caused by spontaneous activity (Ott *et al.*, 1980).

Out of more than 30,000 known species of fish, only five (*Acipenser* spp., *S. marmoratus*, *G. maculatus*, *C. batrachus* and *P. notatus*) are at present thought to be oxygen conformers incapable of maintaining oxygen uptake under moderate hypoxia exposure (Singh & Hughes, 1971; Burggren & Randall, 1978; Graham & Baird, 1984; Urbina *et al.*, 2012; Tripathi *et al.*, 2013). Here we examined the capacity of one of these supposedly unique species, *S. marmoratus*, to regulate their  $\dot{M}_{O_2}$  under hypoxia. In contrast to previous conclusions by Graham (1997) and Graham and Baird (1984), this study supports the suggestion by Bicudo and Johansen (Bicudo & Johansen, 1979), that *S. marmoratus* is indeed an oxygen regulator, with a  $pO_{2crit}$  of  $4.65 \pm 1.59$  kPa. This value is similar to those for many freshwater and marine teleosts [c. 4–5 kPa for 50 species reviewed by Rogers *et al.* 2016 (Ultsch *et al.*, 1978, 1980; Bicudo & Johansen, 1979; Thomas *et al.*, 1988; Chabot & Claireaux, 2008; Thuy *et al.*, 2010)].

Although previous studies have suggested that oxygen conforming occurs in fishes, there are numerous indicators to suggest *S. marmoratus* and other species can regulate oxygen consumption in hypoxia. First, while Bicudo and Johansen (1979) and Graham and Baird (1984) disagreed to whether *S. marmoratus* was an oxygen regulator or conformer, they both found that it begins to rely on aerial respiration at an oxygen tension around 4–8.6 kPa depending on size (Figure 1b). This oxygen tension overlaps with the  $pO_{2crit}$  determined in this study, suggesting that aerial respiration is a natural regulatory response (*i.e.*, oxygen regulation *via* a change in oxygen medium) from the fish adapted for survival in waters with insufficient levels of oxygen to maintain aquatic respiration (Graham *et al.*, 1977; Hughes and Singh, 1971).

A second rationale for the previous suggestion that *S. marmoratus* and other species are oxygen conformers may come from an inappropriate use of respirometry across a large size range of fish (Graham and Baird, 1984). Subsequent findings (Svendsen *et al.*, 2016

a,b) have shown that the respirometer-to-fish-volume ratio has a strong influence on the variation and thus the precision of measured oxygen uptake rate of the fish. This means that, if Graham and Baird used the same size respirometers for all of the different sized fish in their study (information not provided) they would have had significant variances in measurements. For the smallest fish in particular, respirometer-to-fish-volume ratios would have reached an excess of 3000 resulting in very uncertain determinations of  $\dot{M}_{O_2}$  (Svendsen *et al.*, 2016b). An example of this variance can be seen in Figure 1a as an overlap of the SE bars from normoxia down to a pO<sub>2</sub> of 80 mm Hg. Graham and Baird (Graham & Baird, 1984) pooled the data for both large and small fish, which might inadvertently have confounded data. Specifically, the largest air-breathing individuals included in the Graham and Baird (1984) study should be expected to have higher critical oxygen tensions than significantly smaller individuals (Babiker, 1979; Graham & Baird, 1984) and pooling of data points are therefore likely to erroneously portray this species as oxygen conforming.

The definition of SMR is the minimum oxygen uptake of a post-absorptive ectothermic animal required to maintain and sustain life at a given temperature (Beamish, 1964a; Beamish & Mookherjee, 1964). For an oxygen regulator, any deviations below SMR must infer a transition to anaerobic metabolism to fuel the processes of life (Hochachka & Lutz, 2001; Pörtner & Grieshaber, 1993). As anaerobic metabolism will cause a build-up of metabolic by-products such as lactic acid, which is energetically costly to remove from the system (Hochachka & Lutz, 2001), an increase in oxygen consumption during post-hypoxic recuperation will result (Scarabello *et al.*, 1992; Lee *et al.*, 2003). A true aerobic oxygen conformer would lower its metabolism, initially preventing this anaerobiosis (Pörtner and Grieshaber, 1993). For instance, in the study by Urbina and Glover (2012) which suggested *G. maculatus* to be an oxygen



conformer, they also found their experimental fish to show a lactate build-up, indicative of anaerobiosis occurring in progressive hypoxia, as well as showing regulatory responses as increased levels of haemoglobin (Urbina and Glover, 2012) and increased ventilation (Urbina *et al.*, 2011). Lastly, if SMR is not established in normoxia (Urbina *et al.*, 2012; Urbina & Glover, 2013), defining whether a fish is an oxygen regulator or conformer is rationally challenging. It is well known that to establish an accurate estimate of SMR a fish needs to be resting in the respirometer for a considerable time prior to data collection (Keys, 1930b; Snyder *et al.* 2016; Steffensen, 2002; Chabot *et al.* 2016). If measurements in hypoxia are initiated too early during the resting period, or in a period of spontaneous activity, the fish will not be truly resting, its metabolism will be elevated above SMR and it will therefore appear to be an oxygen conformer when the oxygen tension in the water is lowered (Pörtner & Grieshaber, 1993; Ultsch *et al.*, 1981). Spontaneous activity of the fish has previously been attributed as an explanation for the early suggestion that the toadfish *Opsanus tau* (L. 1758) be an oxygen conformer (Hall, 1929) rather than an oxygen regulator as determined by Ultsch *et al.* (1981). The same rationale (Ott *et al.*, 1980) has been applied to the findings for *P. notatus* (Craig *et al.*, 2014; LeMoine *et al.*, 2014). Similarly, *C. batrachus*, an air-breathing fish, has been shown to be an oxygen regulator (Jordan, 1976) as well as a conformer (Singh & Hughes, 1971; Tripathi *et al.*, 2013), although two of these studies documented regulatory behaviour including increased ventilation frequency (Singh & Hughes, 1971; Jordan, 1976) while the third (Tripathi *et al.* 2013) only reported oxygen uptake rate values from a limited range of oxygen levels. Clearly, experimental artefacts are likely to have driven some of the initial erroneous conclusions relating to oxygen conforming in bony fishes.

The results presented here remove one of the last remaining species of bony fish from the list of oxygen conformers and provides support to the theory that all bony fishes are oxygen regulators (Keys, 1930b; Ultsch *et al.*, 1981a; Steffensen, 2006). Only two bony fishes now remain on the list of potential oxygen conformers (*P. notatus* and *G. maculatus*), but tell-tale regulatory responses and anaerobiosis during hypoxia (*G. maculatus*) and possible experimental artefacts (*P. notatus*; Craig *et al.*, 2014; Pörtner & Grieshaber, 1993; Urbina *et al.*, 2011, 2012) also begs the question of whether these species are in fact oxygen regulators.

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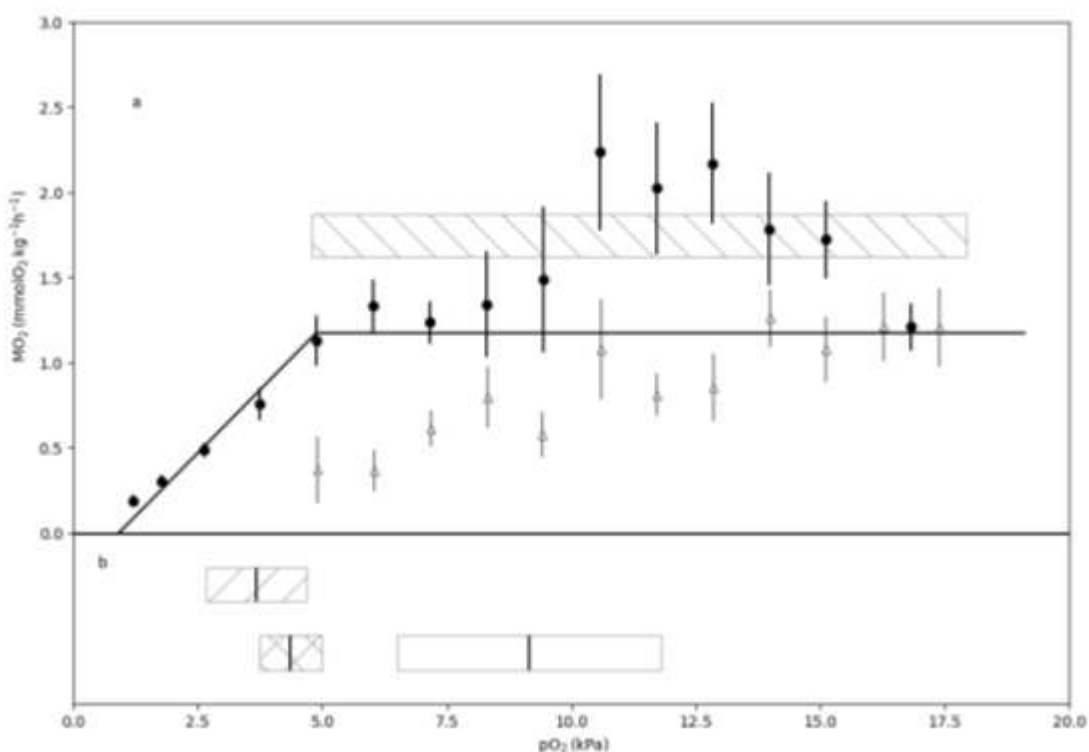
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**Figure 1 (a)** Mean ( $\bullet$ ,  $\pm$  95% C.I.) oxygen uptake ( $\dot{M}_{O_2}$ ) rate *Synbranchus marmoratus* exposed to progressive hypoxia. The standard metabolic rate was found to be  $1.18 \pm 0.14 \text{ mmol O}_2 \text{ h}^{-1} \text{ kg}^{-1}$  (mean  $\pm$  S.D) at a water oxygen partial pressure ( $pO_2$ ) of 18 kPa, and the critical oxygen tension ( $pO_{2crit}$ )  $4.65 \pm 1.59 \text{ kPa}$  (mean  $\pm$  S.D). —, theoretical curve for an oxygen regulator with an SMR of 1.19  $\text{mmol O}_2 \text{ h}^{-1} \text{ kg}^{-1}$  and a  $pO_{2crit}$  of 4.66 mm Hg;  $\triangle$ , the reported values (means  $\pm$  S.E.) from figure 2 in Graham and Baird (1984);  $\square$ , the oxygen consumption rate ( $\dot{V}_{O_2 \text{ water}}$ ) from figure 3 in Bicudo and Johansen (1979). (b) The transition to air breathing for *S. marmoratus*; the top row is from Bicudo and Johansen (1979), average fish mass 117.9 g; the bottom row is from Graham and Baird (1984), average fish mass 151.8 g (hatched) or 722.9 g (unhatched).